Biochem. J. (1986) 240, 601–604 (Printed in Great Britain)

Regulation of lipoprotein lipase synthesis in 3T3-L1 adipocytes by cachectin

Further proof for identity with tumour necrosis factor

S. Russ PRICE,* Thomas OLIVECRONA† and Phillip H. PEKALA*

*Department of Biochemistry, East Carolina University School of Medicine, Greenville, NC 27834, U.S.A., and †Department of Physiological Chemistry, University of Umea, S-90187 Umea, Sweden

We investigated the mechanism by which the endotoxin-induced macrophage secretory protein cachectin is able to suppress the activity of lipoprotein lipase in 3T3-L1 adipocytes. The loss in activity results from an effect on the synthesis of the enzyme, as determined by a decreased incorporation of [^{35}S]methionine into immunoprecipitable lipoprotein lipase. The results were nearly identical whether crude conditioned medium or a highly purified preparation was utilized as a source of cachectin. [^{35}S]Methionine incorporation into acid-precipitable protein was minimally affected by purified cachectin, suggesting that the suppression of the lipoprotein lipase was not due to a general suppression of protein synthesis. These results, taken together with our previous work, provide additional evidence that cachectin and tumour necrosis factor are functionally identical.

INTRODUCTION

Macrophages respond to a variety of invasive stimuli by secretion of monokines. One of these is cachectin, a monokine believed to be responsible for the profound changes in host metabolism during invasive diseases, which led to wasting (cachexia) (Beutler & Cerami, 1986; Beutler *et al.*, 1985*a*,*b*). Among the effects is suppression of lipoprotein lipase activity in adipocytes, which curtails the ability of the adipocytes to extract fatty acids from plasma lipoproteins for storage. Characterization of the cachectin purified from the culture supernatants of RAW 264.7 cells revealed that the 24 *N*-terminal amino acids exhibited a high degree of homology to that of human tumour necrosis factor (Beutler *et al.*, 1985*b*). Moreover, as cachectin also exhibited potent activity in a cytotoxicity assay *in vitro*, Beutler *et al.* (1985*b*) suggested that the two proteins were identical. More recent work with monoclonal antibodies directed against recombinant human tumour necrosis factor has provided evidence that the functional structures of cachectin and tumour necrosis factor are likely to be closely related (Liang *et al.*, 1986), thus providing an additional point of identity between the molecules.

In previous work we have reported that recombinant mouse tumour necrosis factor suppresses the activity of lipoprotein lipase in 3T3-L1 adipocytes at the level of synthesis, on the basis of a loss of ability to incorporate [^{35}S]methionine into immunoprecipitable lipase after exposure to the hormone (Price *et al.*, 1986). The effects of cachectin on several proteins have been traced to decreases in the corresponding mRNAs (Torti *et al.*, 1985). It has not been shown, however, that this is the mechanism by which lipoprotein lipase activity is suppressed. Although generally assumed, there has been no demonstration of an effect of cachectin on the synthesis of lipoprotein lipase at any level. There is evidence that lipoprotein lipase must go through some post-translational activating step to become catalytically active (Ashby *et al.*, 1978; Spooner *et al.*, 1979; Olivecrona *et al.*, 1985; Vannier *et al.*, 1985; Ez-Zoubir *et al.*, 1986). Thus the effect of cachectin on the lipase could be on synthesis or on the putative processing/activating step. To explore this possibility, and to establish an additional critical point of identity with tumour necrosis factor, we have investigated the effect of conventionally purified cachectin on the synthesis of lipoprotein lipase.

EXPERIMENTAL

Cell culture and preparation of cachectin

3T3-L1 preadipocytes were cultured as described by Mackall *et al.* (1976). Differentiation was induced by a modification of the method of Rubin *et al.* (1978). RAW 264.7 macrophages, obtained from the American Type Culture Collection, Bethesda, MD, U.S.A., were grown to confluence in Dulbecco's modified Eagle's medium supplemented with 10% Nuserum. After confluence, the medium was replaced with serum-free medium and the monolayers were incubated in the presence of 0.5 μg of endotoxin/ml of culture medium. After 20 h, the medium was removed, phenylmethanesulphonyl fluoride was added (final concn. 0.1 mM), and the medium was centrifuged at 9500 *g* for 15 min at 4 °C. Cachectin was isolated essentially as described by Beutler *et al.* (1985*b*). From 20 litres of medium, 89 μg of cachectin was obtained. This represented an approx. 200-fold purification, with a yield of about 5%.

Labelling of 3T3-L1 cellular protein

For this, the cells were grown on 3.5 cm dishes, in 2 ml of culture medium. After exposure of 3T3-L1 cells to either conditioned medium from endotoxin-treated macrophage culture (114 μg of total protein) or purified cachectin (25 ng), the cell layers were washed twice with sterile phosphate-buffered saline (0.17 M-NaCl, 3 mM-KCl, 10 mM-Na_2HPO_4, 2 mM-KH_2PO_4), pH 7.4, at room temperature. The monolayers were incubated for 30 min in methionine-free Dulbecco's modified Eagle's

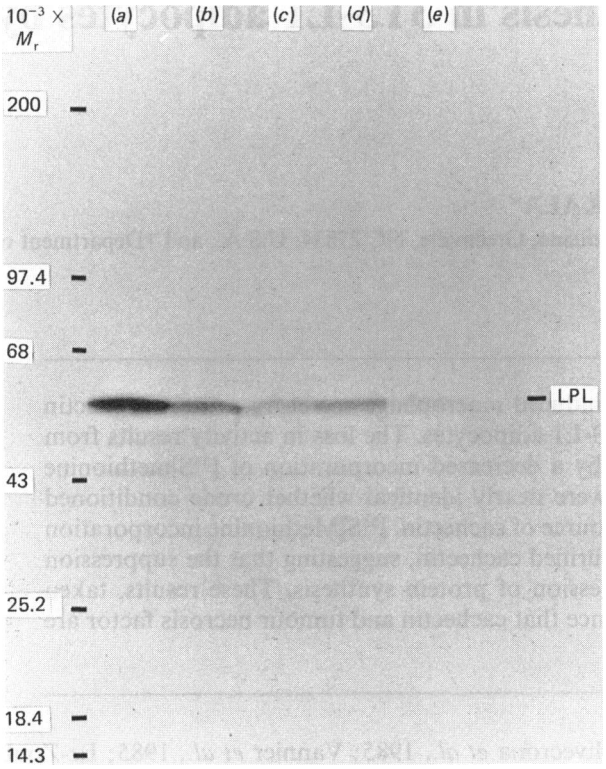

Fig. 1. Suppression of lipoprotein lipase synthesis by crude conditioned medium and by purified cachectin

Lane (*a*), control cells, no exposure to cachectin; lanes (*b*) and (*c*), cells treated with pure cachectin for 3 h and 17 h respectively; lanes (*d*) and (*e*), cells treated with crude cachectin for 3 h and 17 h respectively. Purified bovine milk lipoprotein lipase (LPL) was co-electrophoresed and stained for identification of immunoprecipitated protein. The intensity of the bands was quantified by densitometry. This gave the following relative intensities: control, 100%; pure cachectin 3 and 17 h exposure, 60 and 85% respectively; crude medium for 3 and 17 h, 75 and 90% respectively.

medium under normal growth conditions. Afterwards the medium was aspirated and replaced with 1 ml of methionine-free Dulbecco's modified Eagle's medium containing 75 μCi of [^{35}S]methionine (sp. radioactivity 1200 Ci/mmol) and incubated for 30 min.

Immunoprecipitation of [^{35}S]methionine-labelled lipoprotein lipase

Lipoprotein lipase was immunoprecipitated by the method of Olivecrona *et al.* (1985). The [^{35}S]methionine-pulse-labelled plates were scraped in 1 ml of lysis buffer, consisting of 0.1 M-sodium borate, pH 8.0, 0.5 M-NaCl, 5 mM-EDTA, 10 μg of leupeptin/ml, 1 μg of pepstatin/ml, 3.5 μg of aprotinin/ml and 0.1 mM-phenylmethanesulphonyl fluoride. To 0.81 ml of lysate, 0.09 ml of 10% SDS containing 0.1 mM-phenylmethanesulphonyl fluoride and 5 mM-EDTA was added, and the samples were immediately heated to 95 °C. Portions (0.167 ml) of SDS-treated lysate were diluted with 1.67 ml of 1% Triton X-100 in lysis buffer and allowed to cool. To each sample, 5 μl of normal chicken serum was added, incubated on ice for 2 h, followed by the addition of 150 μl of rabbit anti-chicken IgG serum and incubation at 4 °C overnight. The samples were centrifuged at 1200 ***g*** for 20 min and the supernatants decanted into conical tubes. To the supernatants, either 5 μl of chicken anti-bovine lipoprotein lipase or 5 μl of normal chicken serum was added, and the samples were incubated at 4 °C. After 34 h, 150 μl of rabbit anti-chicken IgG serum was added and the mixture was incubated overnight at 4 °C. The samples were centrifuged at 600 ***g*** for 20 min, the supernatants removed, and the pellets resuspended in 2 ml of 1% Triton X-100/0.1% SDS in lysis buffer diluted 1:10 in phosphate-buffered saline, pH 7.4. After centrifugation at 600 ***g*** for 10 min, the supernatants were discarded and the pellets washed four more times in an identical fashion and once in 62.5 mM-Tris/HCl, pH 6.8. The samples were prepared for electrophoresis by dissolving the pellets in 100 μl of Laemmli (1970) sample buffer. The immunoprecipitated samples were applied to a 17 cm 5–15% linear gradient SDS/polyacrylamide slab

Table 1. Incorporation of [^{35}S]methionine into acid-insoluble protein

3T3-L1 cells were pulse-labelled with [^{35}S]methionine after incubation for various times with either 114 μg of the crude cachectin preparation or 25 ng of purified cachectin, and lysates were prepared as described in the Experimental section. The lysates were all adjusted to a final volume of 1.0 ml, and 10 μl samples were taken to determine [^{35}S]methionine incorporation into protein as previously described (Pekala *et al.*, 1983). Triplicate determinations were performed on duplicate plates. The data reported represent an experiment performed three times with identical results.

Time of treatment	Cachectin preparation	[^{35}S]Methionine incorporated (c.p.m./10 μl of lysate)	Suppression (%)
0 h	(control)	150935±3865	—
1 h	Crude	159525±5769	0
	Pure	153825±12628	0
3 h	Crude	95879±2319	36
	Pure	130915±12313	13
5 h	Crude	84381±622	44
	Pure	126656±9110	16
17 h	Crude	118236±3058	22
	Pure	128389±8049	15

gel and separated by the method of Laemmli (1970). The slab gel was fixed and stained as described by Fairbanks *et al.* (1971). The stained gels were treated with sodium salicylate by the method of Chamberlain (1979), dried and exposed to Kodak X-Omat X-ray film at −80 °C for 72 h and developed. The relative intensity of the bands coincident with immunoprecipitated lipoprotein lipase was quantified with a LKB 220 laser densitometer.

Materials

Lipopolysaccharide B (endotoxin) from *Escherichia coli* 0127 B8 was purchased from Difco Laboratories, Detroit, MI, U.S.A. [^{3}H]Triolein was purchased from New England Nuclear and [^{35}S]methionine was obtained from Amersham. Nuserum was from Collaborative Research, Lexington, MA, U.S.A.

RESULTS

Effects of crude and purified cachectin on lipoprotein lipase synthesis and activity

Addition of either crude or purified cachectin to cultures of 3T3-L1 cells had been shown to result in the suppression of the activity of lipoprotein lipase (Kawakami *et al.*, 1982; Beutler *et al.*, 1985*a*,*b*). To determine if the suppression of the lipase activity was exerted by an effect on the synthesis of new enzyme, and to decide conclusively whether the properties of the crude preparation of cachectin were identical with those exhibited by the purified protein, fully differentiated 3T3-L1 adipocytes were exposed either to conditioned medium (114 μg of protein) from endotoxin-treated macrophage culture or to purified cachectin (25 ng). The amount of protein utilized in these experiments was based on the quantity required to obtain both maximal rates and extents of suppression (> 90%) of lipoprotein lipase activity during the time of the study. After a 17 h incubation the cells were pulse-labelled with [^{35}S]methionine, and lipoprotein lipase was isolated by immunoprecipitation of all lysates (Fig. 1). Exposure to pure cachectin for 3 h (lane *b*) decreased incorporation of [^{35}S]methionine into immunoprecipitable lipoprotein lipase by about 60% relative to control (lane *a*). After 17 h, synthesis of lipoprotein lipase was decreased by 85% (lane *c*). Over this time there was no significant change in general protein synthesis (Table 1). Exposure to the conditioned medium for 3 (lane *d*) and 17 h (lane *e*) resulted in 75 and 90% decreases, respectively, in the synthesis of lipoprotein lipase (Fig. 1). As opposed to pure cachectin, the conditioned medium caused a 40% decrease in general protein synthesis (Table 1); however, by 17 h this appeared to be returning to normal values.

In parallel experiments, the effects on lipoprotein lipase activity were studied (Fig. 2). By 3 h the crude conditioned medium had decreased lipoprotein lipase activity by 75%, and by 17 h the activity was about 90% decreased. Purified cachectin also suppressed lipoprotein lipase activity by more than 90% in 17 h.

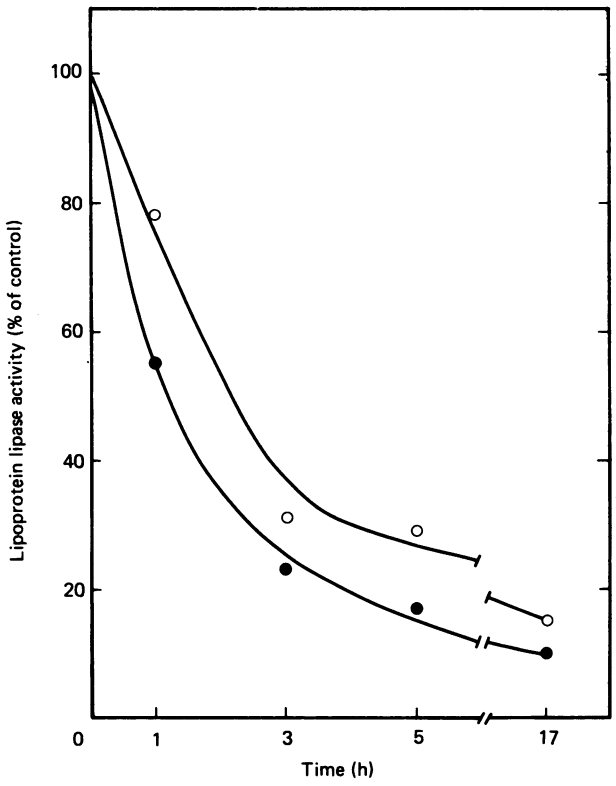

Fig. 2. Time course of the suppression of lipoprotein lipase activity by crude and purified cachectin

3T3-L1 cells were exposed to either (●) crude conditioned medium (114 μg of total protein) or (○) purified cachectin (25 ng of total protein) for various times, followed by determination of the heparin-releasable fraction of lipoprotein lipase activity. These results represent the percentage suppression of lipoprotein lipase activity in treated cells compared with control cells. The lipoprotein lipase was assayed as described by Kawakami *et al.* (1982).

DISCUSSION

Our results demonstrate that the loss of lipoprotein lipase activity in 3T3-L1 adipocytes exposed to either crude or highly purified preparations of cachectin is due to a direct effect on synthesis of the enzyme. Consistent with results from our previous studies (Pekala *et al.*, 1983), in cells exposed to crude conditioned medium there was also a significant decrease in total incorporation of [^{35}S]methionine into proteins. It could thus be argued that the effect on lipoprotein lipase synthesis was part of a general toxic effect on the cells. With purified cachectin, however, there was no general effect on protein synthesis, but synthesis of lipoprotein lipase was more than 90% suppressed. Lipoprotein lipase is known to turn over rapidly; half-lives of about 30 min have been reported for adipocyte-like cell lines (Ez-Zoubir *et al.*, 1986; Wise & Green, 1978). Hence the decrease in synthesis can explain the rapid decline in lipoprotein lipase activity. Moreover, these results, demonstrating the effect of cachectin on the synthesis of lipoprotein lipase with virtually no effect on total protein synthesis, are identical with those observed in our work with purified recombinant tumour necrosis factor (Price *et al.*, 1986). These data further solidify the functional identity between cachectin and tumour necrosis factor.

For our study we used 3T3-L1 cells. These cells, cloned from mouse fibroblasts, differentiate in culture into cells having the biochemical and morphological characteristics of adipocytes (Wise & Green, 1978; Green & Kehinde, 1974). It has been shown that cachectin can block the differentiation, and when added after differentiation can impede transcription of a number of genes

corresponding to differentiation-specific proteins (Torti *et al.*, 1985). We cannot decide from our data if lipoprotein lipase responds as part of this general process, or if there is a direct effect on lipoprotein lipase synthesis. It seems unlikely that the effect of cachectin on adipose-tissue lipoprotein lipase activity *in vivo* is secondary to de-differentiation of adipocytes towards fibroblasts. In any case our results demonstrate that the effect on lipoprotein lipase synthesis is rapid. The synthesis was suppressed by more than 80% within 3 h; this is more rapid than any of the previously reported effects on synthesis of other enzymes (Torti *et al.*, 1985).

This work was supported by NIH grant GM 32892, the North Carolina Board of Science and Technology and the Research Corporation, and by grants from the Swedish Medical Research Council (B13X-727) and from the Lions in Umea Research Fund (399/85).

REFERENCES

Ashby, P., Bennett, D. P., Spencer, I. M. & Robinson, D. S. (1978) Biochem. J. **176**, 865–872
Beutler, B. & Cerami, A. (1986) Nature (London) **370**, 584–588
Beutler, B., Mahoney, J., LeTrang, N., Pekala, P. & Cerami, A. (1985*a*) J. Exp. Med. **161**, 984–995
Beutler, B., Milsark, I. W. & Cerami, A. (1985*b*) Science **229**, 869–871
Chamberlain, J. P. (1979) Anal. Biochem. **98**, 132–135
Ez-Zoubir, A., Vannier, C., Etienne, J. & Ailhaud, G. (1986) Biochim. Biophys. Acta **875**, 334–343
Fairbanks, G., Steck, T. L. & Wallach, D. F. H. (1971) Biochemistry **10**, 2606–2617
Green, H. & Kehinde, O. (1974) Cell **1**, 113–116
Kawakami, M., Pekala, P. H., Lane, M. D. & Cerami, A. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 912–916
Laemmli, U. K. (1970) Nature (London) **227**, 680–685
Liang, C.-M., Liang, S.-M., Jost, T., Sand, A., Dougas, I. & Allet, B. (1986) Biochem. Biophys. Res. Commun. **137**, 847–854
Mackall, J. C., Student, A. K., Polakis, S. E. & Lane, M. D. (1976) J. Biol. Chem. **251**, 6462–6464
Olivecrona, T., Chernick, S. S., Bengtsson-Olivecrona, G., Paterniti, J. R., Jr., Brown, W. V. & Scow, R. O. (1985) J. Biol. Chem. **260**, 2552–2557
Pekala, P. H., Kawakami, M., Angus, C. W., Lane, M. D. & Cerami, A. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 2743–2747
Price, S. R., Olivecrona, T. & Pekala, P. H. (1986) Arch. Biochem. Biophys., in the press
Rubin, C. S., Hirsch, A., Fung, C. & Rosen, O. M. (1978) J. Biol. Chem. **253**, 7570–7578
Spooner, P. M., Chernick, S. S., Garrison, M. M. & Scow, R. O. (1979) J. Biol. Chem. **254**, 10021–10029
Torti, F. M., Dieckmann, B., Beutler, B., Cerami, A. & Rignold, G. M. (1985) Science **229**, 867–869
Vannier, C., Ez-Zoubir, A., Etienne, J., Negrel, R. & Ailhaud, G. (1985) J. Biol. Chem. **260**, 4424–4431
Wise, L. S. & Green, H. (1978) Cell **7**, 233–242

Received 11 September 1986; accepted 3 October 1986